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THE STUDY OF COLONY FORMATION IN DEEP AGAR *

STUDIES ON PATHOGENIC ANAEROBES VI

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Under uniform conditions living creatures of the same species vary in shape only within comparatively narrow limits. Morphology is, in the case of the majority of living things, the character that is the most easily observable, and for this reason it has been used as a basis for the classification of all forms of life. Among the bacteria such use of morphologic criteria for systematic purposes is frequently avoided because the form of these organisms is exceedingly simple and because the bacteria are highly specialized, not as to shape but as to the enzymes with which they split the substances surrounding them. Higher plants are classified not according to the shape of their stems and leaves, but according to that of their reproductive organs. For purposes of bacterial classification, we may well let chemical characters take the place of those morphologic ones that the bacteria do not possess. A chemical classification will then serve to build a skeleton structure which will group and divide the bacteria into genera¹ that are comparable with those of the higher plants. The trend followed in dividing the bacteria has usually been to employ morphologic characters for the making of major divisions and chemical ones for the finer differentiations. For many reasons pointed out in a former paper² morphologic criteria fail to furnish a means for logically subdividing the major group of anaerobic rods. Chief among these are the facts that most morphologic characters appear in various groups, that there is a general overlapping of such characters and that there is a lack of correspondence between chemical and morphologic differ-

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* The present paper is intended as an introduction to others in which the colony formation of anaerobic strains will be discussed.

¹ Heller, H. H.: J. Bacteriol., 1922, 7 (Jan.).

² Ibid., 1921, 6, p. 521.

ences. It is the purpose now to indicate the exploitation of a so-called morphologic character for purposes of finer differentiation.

Anaerobic bacilli form within a solid nutrient agar mass colonies of very different shapes, but many types are constant in their behavior if the conditions surrounding the organisms are uniform. This constancy is such that if a strain of anaerobic bacilli be inoculated, on various occasions, from a culture of the same age grown on a similar medium, always onto the same variety of deep agar, and if it be incubated for the same period at an identical temperature, it will produce colonies that are enough alike to furnish the systematist with an excellent means for the comparison and identification of cultures. This constancy is such that the slight variations in conditions that occur when technic is faithfully imitated by conscientious workers in different laboratories are not sufficient seriously to impair the use of deep colonies for systematic purposes in certain of the anaerobic groups when simple mediums are employed. The colonies are similar enough to warrant their comparison to the leaves and stems of higher plants. The same overlapping of characters of vegetative portions appears among higher plants as among bacterial colonies. Thus some senecios look more like cacti than they do like other senecios; the foliage of a hakea resembles that of some accacias more than that of other hakeas; remarkable resemblances of foliage occur among members of the genera *Juniperus*, *Cupressus* and *Chamycyparis*. But sort out the senecios and the hakeas on the basis of the morphology of their reproductive organs, then their leaves and stems differentiate their species from each other; sort out the bifermentans from the Welch bacilli and the vibriion septique from the sporogenes on the basis of their chemical reactions, and the morphology of their colonies will serve to group strains that have thus been sorted into genera into smaller groups which may or may not be considered as species, depending on the constancy of colony formation in those genera. The only great difference in such a comparison between the higher plants and the bacteria is that the latter, immersed in gels containing liquids of widely different composition, are far more subject to such variation as is determined by external conditions than are the former.

Surface colonies of aerobic organisms have been used for systematic purposes and for the study of mutations for many years. Such colonies are frequently characteristic in shape, but colony characters other than form, such as pigment formation and opacity, have been frequently

used for these types of investigation. The aerobic surface colony is much more characteristic and uniform in contour than the anaerobe surface colony. The former is automatically limited in size because grown in the dry air of the incubator, while the latter is subject to such different moisture and gas conditions that though specifically fairly characteristic, it is not very uniform in behavior. Anaerobic surface colonies have been described in most detail by McIntosh,³ Henry,⁴ Adamson⁵ and Zeissler.⁶ The latter has used colonies on glucose blood agar as a basis for the subdivision of the vibrión septique group;⁷ his method has the advantage of showing hemolysis. In the depths of an agar medium gas and moisture conditions are so controlled that their disturbing effects are eliminated, and colonies growing therein are more uniform in behavior, and their study permits much more accurate determination than does that of surface growths.⁸ Deep colonies have been figured most extensively by von Hibler⁹ and by Weinberg and Seguin.¹⁰

Illustration of the way in which certain of the anaerobic genera may be subdivided by colony determination will be given in subsequent papers. A collection of colony photographs of organisms that do not belong in the groups to be later discussed is included in the present paper (plates 1-5).

The shape of a bacterial colony is not to be thought of as a matter of chance nor, on the other hand, is it in any degree so dependent on the inherent germ plasm as are the shape and histologic arrangement of the tissues of the higher plants. It is dependent, first, on the moisture content of the medium, secondly, on the texture of the colloid mass as determined by the rapidity of cooling, thirdly on the ability of the organism to multiply in the medium, and fourthly on the motility of the organism in the medium. Intrinsically characteristic generic or specific colony form, if it exists, is subordinate to these four factors. All the chemical phenomena that are observable in bacterial growth in liquid medium must be thought of as contributing to shape the colony in a solid medium. *Pabulum*—split proteins, sugars and vitamins;

³ Med. Research Committee, Special Report 12, 1917.

⁴ J. Path. & Bacteriol., 1917, 21, p. 344.

⁵ Ibid., 1919, 22, p. 345.

⁶ Ztschr. f. Infektionskr. d. Haustiere, 1920, 21, p. 1.

⁷ Heller, H. H.: J. Infect. Dis., 1921, 27, p. 385. Reprints, Hooper Foundation, 1921, 6.

⁸ Heller, H. H.: J. Bacteriol., 1921, 6, p. 521.

⁹ Ueber die pathog. Anaeroben, 1908.

¹⁰ La gangrène gazeuse, 1918.

lag; initial reaction, buffers, acid inhibition, neutralization by alkaline end-products. Temperature, acid, and autointoxication have their effect on motility as well as on growth.

Therefore the acquisition by an individual bacillus of the ability to split and assimilate a nutritive element present in a substratum will make itself known by an increase in the size of the colony formed by the mutating bacillus. Likewise the loss of a power involved in the assimilation of nutriment from a medium will cause the formation of smaller colonies in that medium. Other mutations involving the formation of autointoxication products, the motility, or even the rate of disintegration of bacillary bodies, will also affect the size or transparency of the colonies.

Colony shape may then be used in the case of anaerobic bacilli as a technical means of spotting mutations. Mutating forms may then be isolated, studied on the medium in which they appeared, and suitable experiments may then be arranged to demonstrate, if possible, what was the metabolic cause of the change in colony form.

As emphasized before,² the mutations of anaerobes, though little studied, cannot be essentially different from those of other living things. Careful work makes it seem highly improbable that such mutations ever involve many characteristics at once. The anaerobic bacilli are relatively stable types. A Welch bacillus cannot be changed into an amylobacter, nor a blackleg bacillus into a Welch bacillus or sporogenes bacillus or aerobic bacillus. On the other hand, to claim that mutations do not occur among these organisms would be to state that they lack one of the best recognized attributes of living matter. So far as we know, they have originated by descent and not by spontaneous generation—if not by mutation, then how? Apparently mutation, such as may be recognized by a change in colony form, occurs far more frequently in some groups of anaerobic organisms than in others. In such groups colony form is not available for systematic purposes.

A colony differing from its neighbors, when fished onto a new series of agar tubes, may or may not give new colonies like itself. For the observation of such colonies a control series of the normal type present should always be inoculated, and the appearance of the colonies should be noted at several different time intervals. Occasionally two colonies will be found in a culture derived from a single colony, whose descendants pass through the same morphologic history,

but do so at consistently different speeds. There is, then, a difference between these strains, and the new form may be accepted as a mutant of the other. On the other hand, one may often find two colonies of different appearance from a tube containing a considerable number of colonies, and their offspring may be all alike or show similar variations that are clearly not mutations. One more set of dilution tubes will usually be sufficient to settle the question whether a mixture of two types is present or whether the differences noted lie within the limits of variation for the species. Some anaerobic strains occasionally show in thickly seeded tubes one or a few colonies several times larger than the others. These are usually constellations of small colonies derived from minute chunks of infected inoculum. When planted on a new series of tubes, the same forms may appear in about the same proportion as before, and repeated selection from such colonies does not increase their number. These large colonies do not breed true and are thus not mutations.

TECHNICAL CONSIDERATIONS INVOLVED IN THE STUDY OF ANAEROBIC COLONIES

Inoculation of material for photographic representation of the colonies hereafter pictured was as follows: Liquid from meat cultures was used for inoculum. In the case of rapidly growing anaerobes one or two day cultures only were employed. In the case of proteolytic organisms a somewhat older culture was occasionally used, but old run out cultures were avoided. Tubes of agar were melted in the water bath and were then tapped and shaken sharply and replaced in the water bath for a few minutes and then shaken again. This method removes the air without soaking the plugs with steam. The tubes were then cooled to 42 C., and the first was inoculated with a small loopful of culture and shaken; the second was usually inoculated from the first with the amount of agar that would fill the capillary of a large bore Pasteur pipet; the pipet was then well flamed, and the third or third and fourth tubes were inoculated with somewhere between 0.25 and 0.75 c.c. of material from the second tube. Colonies were well distributed by rolling and tipping. The tubes were allowed to cool in air at room temperature.

Twenty-four hours was chosen as the standard time for incubation unless there was a reason for choosing another period. Thus blackleg colonies are invisible at 24 hours, as are those of some strains of tetanus. For some organisms a comparatively early stage is characteristic and for others a later stage is more so.

Preparation of colonies for photographing is carried out as follows: The tube containing colonies to be photographed is held with the mouth slanting toward an open Petri dish, and an open large bore Pasteur pipet bent at a right angle is inserted along one side to the bottom. This pipet is then removed and the agar blown out of it, and it is then reinserted and through it the column of agar in the culture tube is blown out into the Petri dish. A safety razor blade may be used to section the medium, usually at right angles, and

slices about 2 mm. thick are placed on a slide with a drop of water and covered with a small coverslip. These are examined directly on the ground glass of the camera.

Photographic Technic: The microscope and camera are arranged on the plan designed by Dr. L. B. Wilson,¹¹ the lens of the camera being removed. The source of illumination for most of the pictures was a Spencer light placed under the stage of the instrument. Mirror and condenser were removed. A ground glass disk such as accompanies Leitz microscopes was placed on the stage under the slide. For later work a nitrogen light¹² and a mirror without condenser have been employed. The ocular used in all cases has been a Zeiss projection ocular No. 4, and two objectives have been employed, Zeiss No. A₂ arranged to give on the ground glass of the camera a magnification of 15 diameters, and a Leitz No. 3 which gives an enlargement of 50 diameters. For work in which a wide range of lighting conditions must be recorded, such as one finds in general colony work, Seed's "Non-halation-L-Ortho" plate has been found superior, and Seed's "26" plate is also excellent. These two are fairly rapid and give, apparently, a maximum of detail in contour and in the minute fuzzy halos surrounding many colonies. For representation of the interior structure of smooth-contoured colonies, Dr. K. F. Meyer tells me that he finds "Process" plates excellent. Their range is not sufficient for general work. Seed's "23" plate also gives too much contrast for most purposes. Orthonon plates were found to be too thin. All the exposed plates were developed in a tank according to the directions accompanying them. It is well to choose a type of emulsion that may always be had and to use that type constantly for comparative work. Regular glossy Velox paper was used for the prints.

All conditions regarding age of colonies and age of inoculum, medium, incubation temperature and photography, are made as uniform as possible and are carefully recorded.

Contamination.—For ordinary laboratory purposes it is sufficient for the investigator of anaerobes to inoculate his cultures in a small quiet room where no other people are moving about. For a worker with considerable practice and skill contaminations occur rarely under such conditions, at least in an atmosphere that contains little dust. It is hopeless to try to do accurate work in a busy room.

Crowding.—The number of colonies present in a tube has a striking effect not only on the size but also on the shape of the colonies in the tube. Many types of colonies originate as lenticular masses and then send out protuberances or downy strands. If colonies with such a habit are abundant, they pass through the various morphologic phases in a shorter period of time than if they are few, and different colonies in the tube pass through their development at different speeds, so that from a single tube with many colonies the entire history of colony

¹¹ Spencer photomicrographic outfit 4336B.

¹² Bausch and Lomb adjustable microscope lamp No. 1784.

formation may be traced. This point is illustrated by the series of photographs showing the process of colony formation of a *Rivoltillus*¹ (vibron septique) strain (Plate 6). Where colonies are numerous they are, of course, smaller than where they are few. For these two reasons therefore, types should always be chosen from tubes with so few colonies that they do not interfere with each other. A striking case of difference not only in form of the colony but in its texture is shown by the series of photographs of colonies of an unidentified anaerobic organism (Plate 7). To designate the number of colonies present in a tube, I have usually employed the following rough scale:

Sole colony	1
Colonies few	1— 20
Colonies not numerous.....	20— 60
Colonies moderately abundant.....	60—400
Photographed for history of development	
Colonies, abundant	over 400
Rarely photographed	

Permeating Growth.—The more rapidly proliferating species of anaerobes, motile and nonmotile, frequently leave their colonies and multiply in the agar gel as though it were a broth.⁸ When this happens the colonies abruptly stop growing. Tubes containing this permeating growth may be readily identified by holding them to the light with a control. When there is more than a suspicion of this phenomenon the colonies should not be used for systematic measurements unless the permeation of the medium is a constant character of the strain. Plate 8 shows the effect of permeating growth on the size of the colonies. Colonies in agar cooled rapidly in water or ice water are more sharply delimited than those in agar that is cooled at room temperature. Uniform procedure should be adopted with regard to cooling the agar, or if the procedure be varied to diminish permeating growth note should be made of the fact.

Uniform Conditions.—An instance may be given of the difficulties encountered by the student desirous of identifying an anaerobic strain. No worker has time to duplicate the mediums and technic of von Hibler,⁹ who gives excellent photographs of colonies in his book. Plate 2 of his book may be taken as an example. Among the mediums used for the growth of the colonies represented on this page are glucose cat-meat agar, spleen-broth agar, sodium formate spleen-broth agar, rabbit-meat agar, guinea-pig-meat agar, and for illustrations on other plates fish-meat agar and various other queer concoctions have

been employed. Far better be it to give the conditions of growth as von Hibler does than not to give them at all. One appreciates his effort to study the organisms under different conditions, but he made his work hopeless to follow. He gives us a single page with 4 pictures of the morphology of 3 species of bacteria in the animal body. Then come 12 photographs of deep colonies and two of surface colonies of 8 species of organisms. These are photographed at 10 different ages ranging from 10 hours to 3 days, the organisms have grown on 9 different mediums from 4 or more different kinds of animals (it is to be hoped that he never confused his mediums) represented in 7 different enlargements. The anaerobist working on bibliography is surely a creature to be pitied, and is not to be blamed for failing to follow the work of others conscientiously and thoroughly. Von Hibler is also to be criticized for terming colonies formed on one kind of medium "typical" and on another kind "atypical."

Photographs of colonies should always be accompanied by the name of the strain that gave rise to them. The beautiful lithographs of oedematiens colonies shown by Weinberg and Seguin¹⁰ represent colonies very different in aspect, but there is no indication which forms are characteristic for the various strains that another worker might consider to belong to distinct species.

One photograph is usually insufficient to give a fair idea of the possible variations of a strain. Several colonies should be depicted, the number depending on the limits of variation in the strain.

The medium chosen should be one on which as many species as possible will grow, but a universally suitable one is probably not to be hoped for. Liver agar has been found eminently satisfactory for most types of anaerobes but affords too fertile a substratum for some. It was excellent for producing a suitable colony formation with black-leg, oedematiens, tetanus and botulinus organisms. *Vibrio septique* strains grew too rapidly in it so that at 24 hours colonies descended from spores were smaller than those descended from vegetative rods; at 48 hours, they were uniform in size but permeating growth was so frequent that many tubes had to be discarded, and the growth was so luxuriant that the colonies of different strains could not be so nicely grouped as in the case of other genera. A search should be made in such cases for a less nutrient substratum or for a firmer medium. But there are indications that liver agar is so complex a medium that it furnishes too much chance for mutations to affect the shape of the colonies, and simpler mediums may have to be adopted. Agar more

than a month old should not be used for colony photographs as it is too dry. For most types of anaerobes sufficiently uniform colonies may be secured on medium from 1 to 30 days old. Blackleg strains will not grow after the medium is from 6 to 10 days old. Agar that is too fresh or that contains too much moisture will also give misleading results (Plate 7). Such agar may be incubated to dry it out before using.

Liver Agar.—This is made up of beef liver 1 part, distilled water 4 parts. Infuse over night in the refrigerator, bring to a boil, strain through cheesecloth, add 1½% Difco peptone, 0.5% salt and 2% agar. Melt the agar, titrate and adjust to P_H 7.2, cool to 60 degrees, add white of egg and serum; autoclave for one hour, titrate again and adjust to P_H 7.2; filter through cotton, tube, and sterilize in the autoclave.

CONCLUSIONS

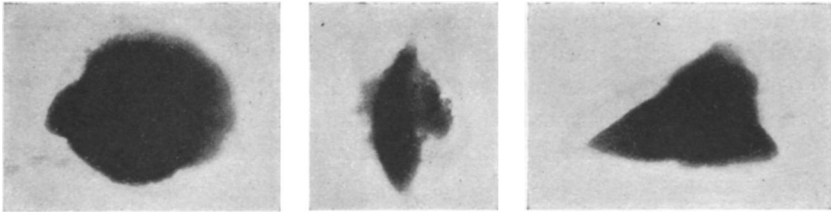
Under constant conditions members of a pure strain (biotype) of anaerobic bacteria make deep colonies that differ from each other scarcely more than do the vegetative portions of individuals of a species of higher plants.

It is found that a collection of anaerobic bacilli that correspond in all ordinary cultural characteristics may be broken up into groups of strains on the basis of colony formation in deep agar. These groups are, in some genera, when the medium employed is simple, to be termed "species," in others not. The observation of colonies may thus be of great value to the systematist.

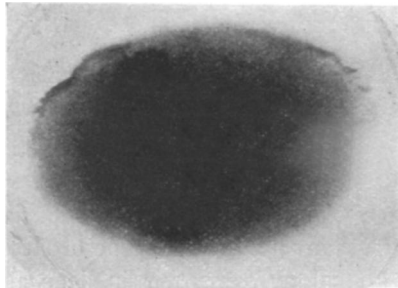
The shape of a colony in an agar medium depends primarily on the reproductive power of the organism and its motility in that medium. The reproductive power depends on its enzymes and metabolic activities. Therefore, any mutation involving the metabolic activities of the organism on a given medium may demonstrate its occurrence by causing a change in the colony morphology of the mutating organisms in the strain. Colony formation in deep agar is, then, a phenomenon whose study will be most closely observed by the student of mutation.

The colony shape must thus be thought of as a chemical character as well as a morphologic one, and the possibilities of its exploitation on specially prepared mediums to test the nature of mutations are infinite.

Technical considerations and various pitfalls are discussed: preparation of colonies and their photography, contamination, crowding, permeating growth, uniform conditions and choice of medium.



Bacillus 12 of McIntosh, type strain. Colonies not numerous; $\times 50$.



An unidentified mildly proteolytic organism isolated from stools of infants by Miss J. Easton. Colonies few; $\times 50$.

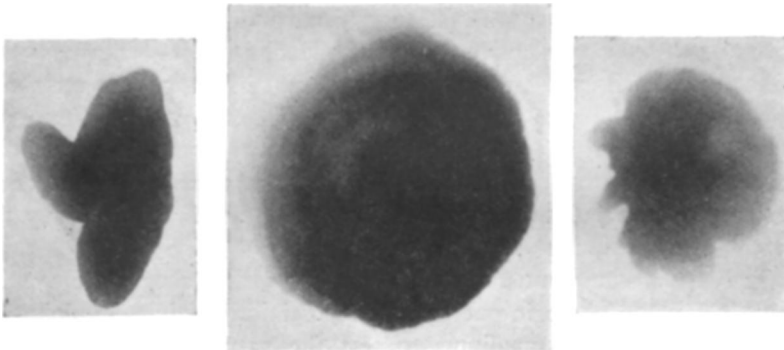
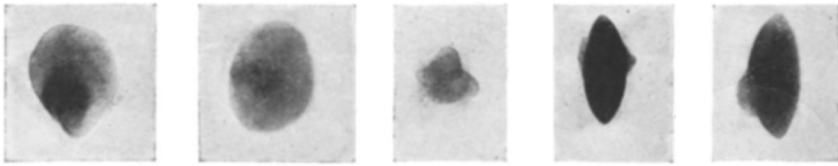
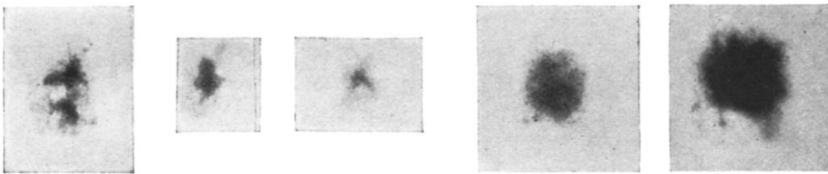


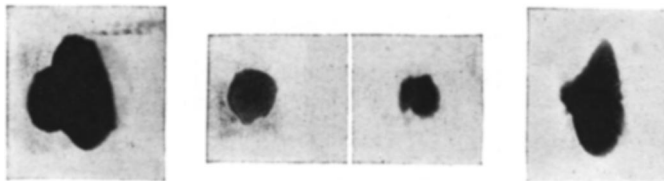
Fig. 1.—Lenticular and modified lenticular types. Except where otherwise noted, the colonies photographed in this series were grown on beef liver peptone agar for 24 hours at 37 degrees. The nomenclature employed is explained in another paper.¹



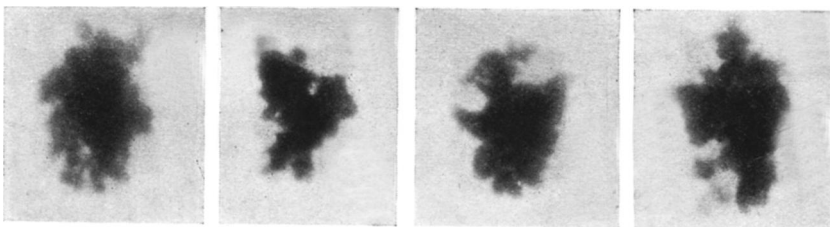
Putrificus sp.; strain IOR from septic wound. This corresponds more nearly to Bientstock's description of *B. putrificus* than any other strain in the collection. Colonies moderately abundant; $\times 50$.



Many colonies present, 18 hours. Few colonies present, 24 hours.
Weinbergillus histolyticus, type strain, HW; $\times 50$.

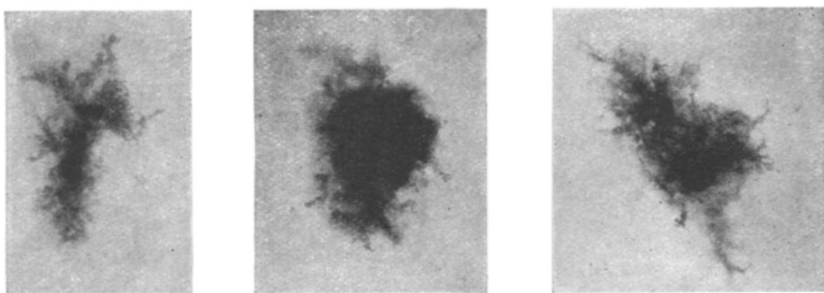


Seguinillus aercoetidis, type strain, AW. Colonies few; $\times 15$.

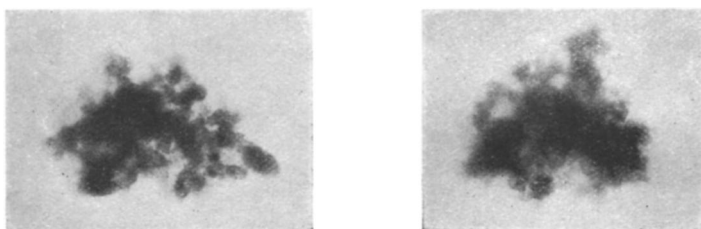


Vallorillus fallax, type strain, FW. Colonies few, 4 days old. Note diffuseness of outline due to permeating growth; $\times 50$.

Fig. 2.—Colony forms.

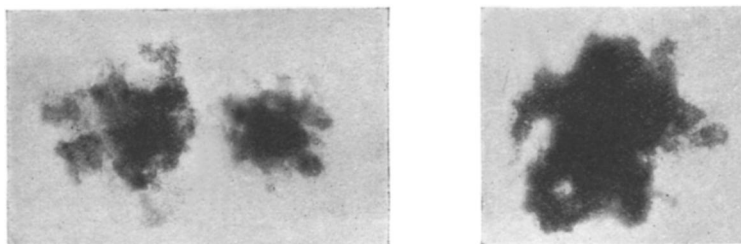


Martellillus proteolyticus; type strain, F, from septic wound, man. Colonies few; $\times 50$.

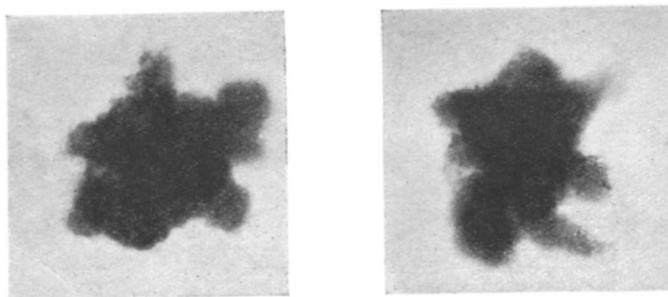


Martellillus sp. Strain JC, from Bradsot material. Conditions as above.

THE BIFERMENTANS TYPE

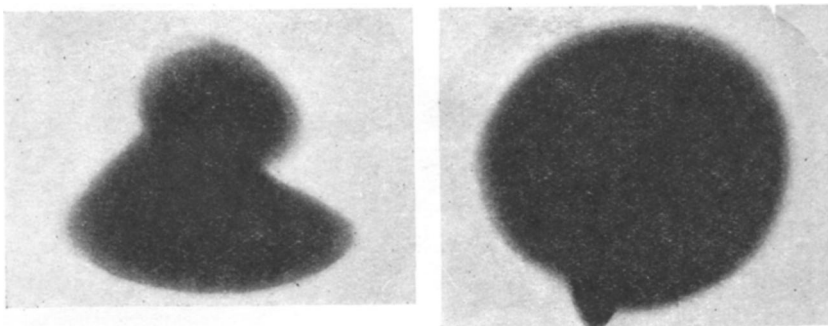


Eighteen hours, colonies few.



Twenty-four hours, colonies moderately abundant.
Robertsonillus primus. Type strain, L, from septic wound, man; $\times 50$.

Fig. 3.—Colony forms.

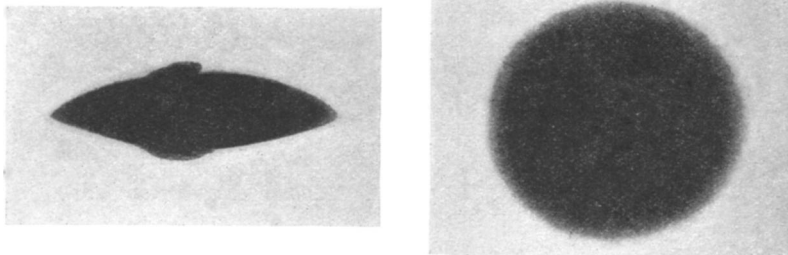


Strain WM; few colonies.

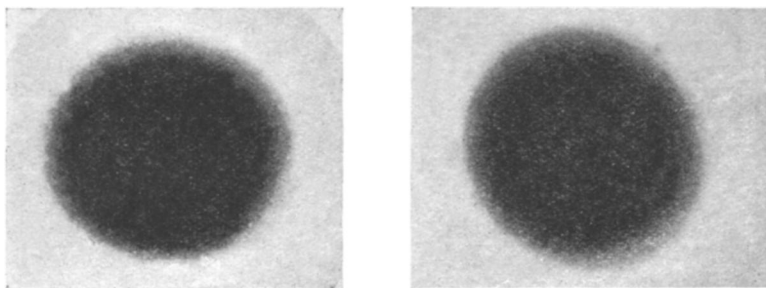


Strain WAT; few colonies.

Welchillius (*B. Welchii*) colonies; $\times 50$. Note dulness of outline due to permeating growth.

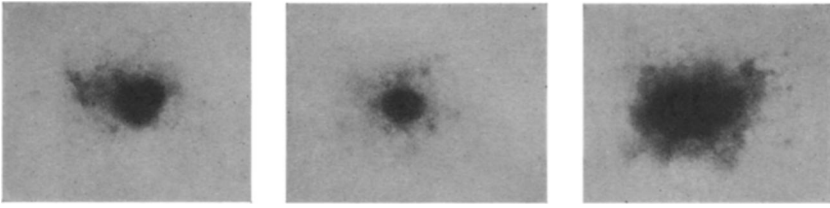


Strain P 11; isolation of Robertson; few colonies.

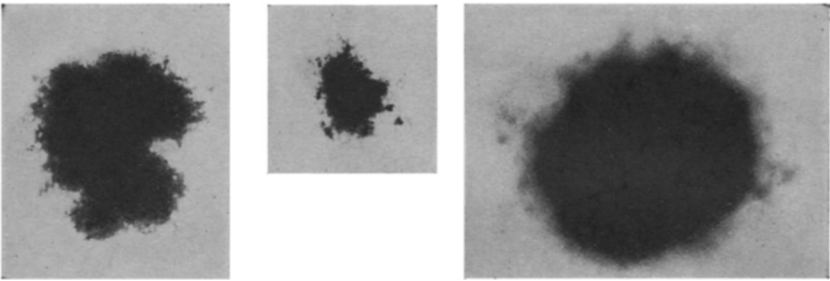


Strain P 7; isolation of Robertson; few colonies. Henrillus (*B. tertius*) colonies; $\times 50$.

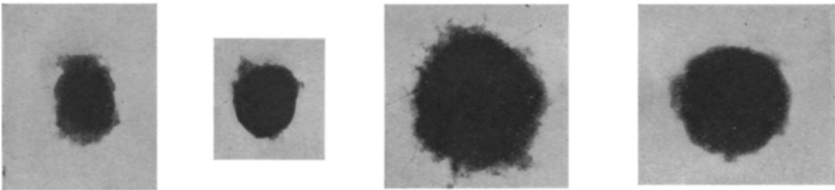
Fig. 4.—Nonmotile organisms.



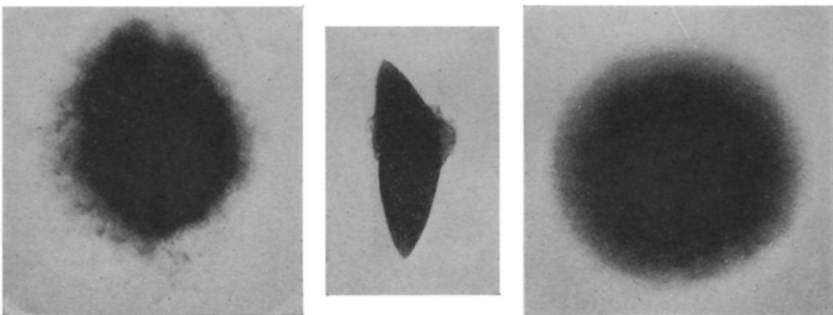
Eighteen hours old, colonies not numerous; $\times 50$.



Two days old, not numerous; $\times 15$. Note difference in size of colonies. $\times 50$. Veal infusion agar; eighteen hours; Metchnikovillus sporogenes; Strain MR; isolation of Robertson.

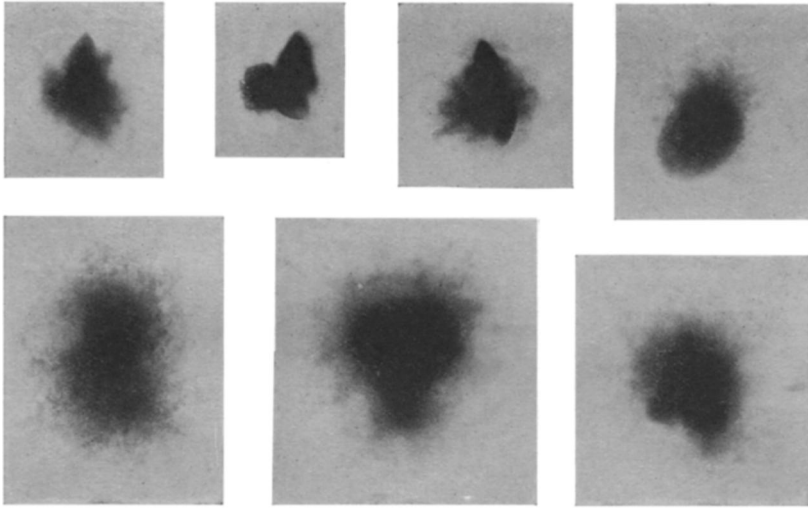


Eighteen hours; $\times 50$. Eighteen hours, veal infusion agar. Metchnikovillus parasperogenes McIntosh; strain R 60.

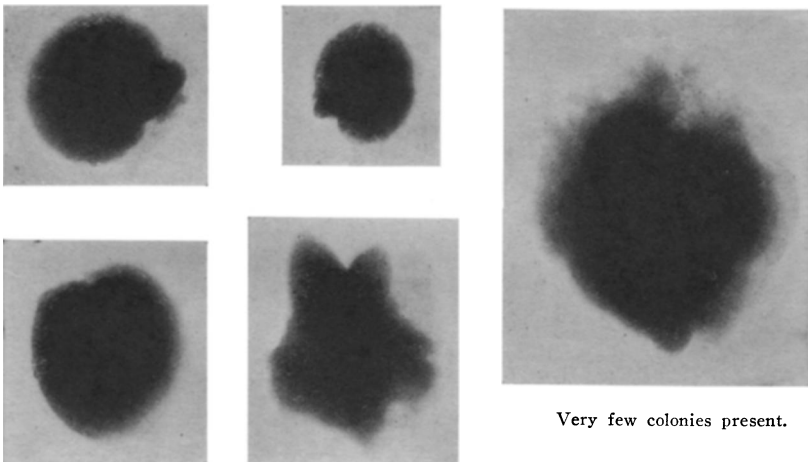


A culture of "Malignant oedema bacillus" from Germany resolved itself into two species, a typical sporogenes strain (at the left) and a nonmotile less strongly proteolytic organism (at the right).

Fig. 5.—Highly motile organisms.

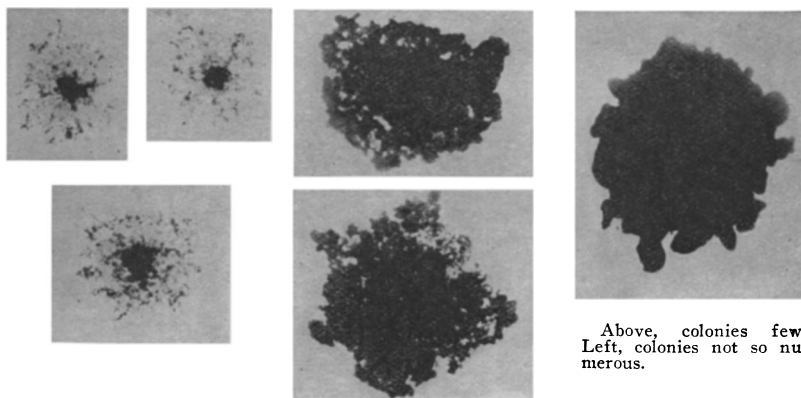


Colonies moderately abundant.



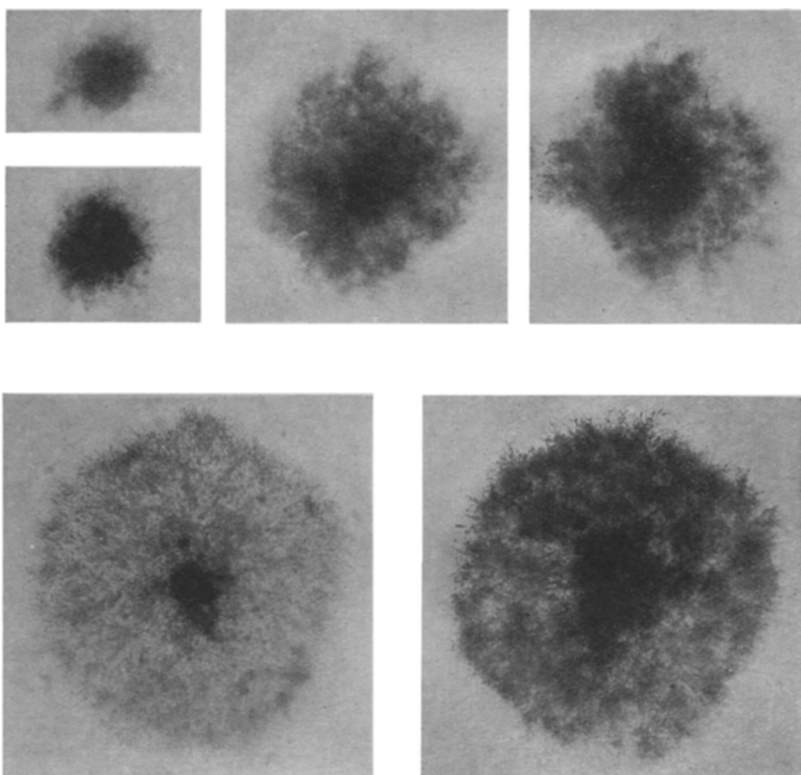
Very few colonies present.

Fig. 6.—The development of a vibron septique colony. Vibron septique strain VS that retains its lenticular form unusually long (24 hours); $\times 50$. The influence of crowding is well shown. The second group of colonies developed in a tube inoculated from the first. A greater dilution of inoculum was the only difference in the conditions in the two tubes.



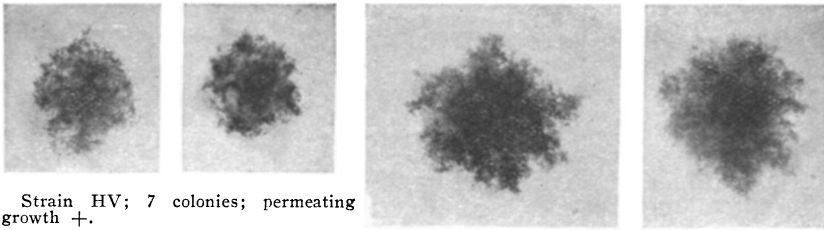
Above, colonies few.
Left, colonies not so numerous.

An extreme example of the influence of crowding. An unidentified organism isolated by Miss J. Easton. Three successive dilutions of a pure culture; $\times 50$.



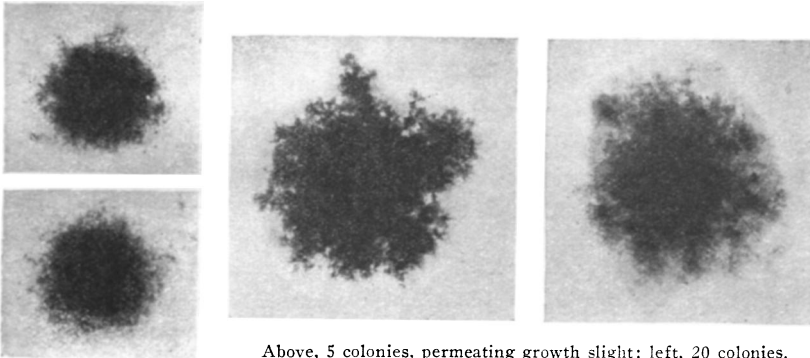
An instance of the influence of moisture in the agar on colony formation. Tetanus strain T 087, grown under otherwise identical conditions in "2%" agar that was (above left) old and dry (above right), fresh, and (below) unusually soft.

Fig. 7.—Influence of crowding and of moisture.



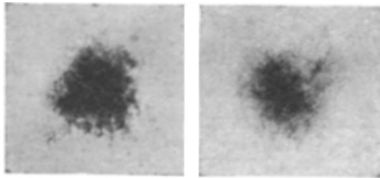
Strain HV; 7 colonies; permeating growth +.

Eight colonies; permeating growth —.

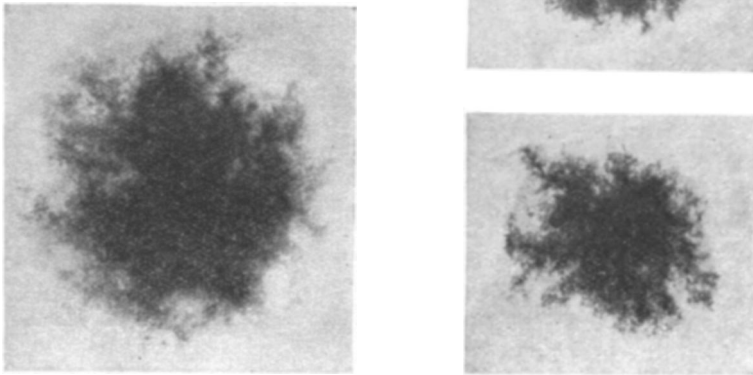


Strain AS

Above, 5 colonies, permeating growth slight; left, 20 colonies, permeating growth ++.



Strain BN; 3 colonies; permeating growth slight.



Sole colony, permeating growth —. Twenty colonies, permeating growth —.

Fig. 8.—The influence of permeating growth on the size of the colony. *Vibrio septique* colonies 48 hours old; $\times 50$.